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Virus growth and antibody responses following respiratory tract infection of ferrets and mice with WT and P/V mutants of the paramyxovirus Simian Virus 5

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ABSTRACT

P/V gene substitutions convert the non-cytopathic paramyxovirus Simian Virus 5 (SV5), which is a poor inducer of host cell responses in human tissue culture cells, into a mutant (P/V-CPI-) that induces high levels of apoptosis, interferon (IFN)-beta, and proinflammatory cytokines. However, the effect of SV5-P/V gene mutations on virus growth and adaptive immune responses in animals has not been determined. Here, we used two distinct animal model systems to test the hypothesis that SV5-P/V mutants which are more potent activators of innate responses in tissue culture will also elicit higher antiviral antibody responses. In mouse cells, *in vitro* studies identified a panel of SV5-P/V mutants that ranged in their ability to limit IFN responses. Intranasal infection of mice with these WT and P/V mutant viruses elicited equivalent anti-SV5 IgG responses at all doses tested, and viral titers recovered from the respiratory tract were indistinguishable. In primary cultures of ferret lung fibroblasts, WT rSV5 and P/V-CPI- viruses had phenotypes similar to those established in human cell lines, including differential induction of IFN secretion, IFN signaling and apoptosis. Intranasal infection of ferrets with a low dose of WT rSV5 elicited ~500 fold higher anti-SV5 serum IgG responses compared to the P/V-CPI- mutant, and this correlated with overall higher viral titers for the WT virus in tracheal tissues. There was a dose-dependent increase in antibody response to infection of ferrets with P/V-CPI-, but not with WT rSV5. Together our data indicate that WT rSV5 and P/V mutants can elicit distinct innate and adaptive immunity phenotypes in the ferret animal model system, but not in the mouse system. We present a model for the effect of P/V gene substitutions on SV5 growth and immune responses *in vivo*.

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Introduction

Members of the paramyxovirus family of negative strand RNA viruses employ a diverse range of mechanisms to circumvent host cell antiviral responses, including limiting cytokine and type I interferon (IFN) synthesis, blocking IFN signaling pathways and inhibiting apoptosis (reviewed in [Conzelmann, 2005](#); [Garcia-Sastre, 2001](#); [Goodbourn et al., 2000](#); [Horvath, 2004](#)). Many of these mechanisms for counteracting cellular responses have been attributed to products of the P/V gene which typically encodes both the phosphoprotein P subunit of the RNA-dependent RNA polymerase ([Kolakofsky et al., 2004](#)) and the V protein which counteracts antiviral responses ([Didcock et al., 1999a,b](#); [Parisien et al., 2001](#)). For some paramyxoviruses, the P/V gene also encodes the family of multifunctional C proteins that are involved in suppressing antiviral responses and in controlling viral gene expression and virion release ([Devaux and Cattaneo, 2004](#); [Garcin et al., 2001](#); [Lamb and Parks, 2007](#)). For Simian Virus 5 (SV5), accurate transcription of the P/V gene results in mRNA that codes for the accessory V protein. The P mRNA is

identical to the V mRNA except for the addition of two nontemplated G residues that are inserted by the viral polymerase at a precise location in the P/V transcript ([Thomas et al., 1988](#)). Thus, the SV5 P and V proteins are identical for the 164 amino-terminal residues (the shared P/V region), but differ in their C-terminal sequences. The P and V proteins have unique C-terminal domains, with the V protein encoding a highly conserved cysteine-rich (cys-rich) zinc-binding domain that is required for many V-associated functions ([He et al., 2002](#); [Paterson et al., 1995](#)).

A major function of the SV5 V protein is the inhibition of IFN synthesis and signaling ([Childs et al., 2007](#); [Didcock et al., 1999a,b](#); [Poole et al., 2002](#)). During infection of a wide range of animal cells, V protein forms a cytoplasmic complex that directs the ubiquitylation and targeting of STAT1 (signal transducer and activator of transcription 1) for degradation ([Andrejeva et al., 2002](#); [Ulane et al., 2005](#)). Recently, the SV5 V protein has also been shown to block activation of the IFN-beta promoter ([He et al., 2002](#); [Poole et al., 2002](#)), through V protein targeting the IFN-inducible RNA helicase mda-5 ([Childs et al., 2007](#)) by binding with the cys-rich region ([Andrejeva et al., 2004](#)). Thus, the multifunctional V protein counteracts IFN responses at two steps, resulting in both limited induction of IFN synthesis and a block in IFN signaling.

In addition to the cys-rich C-terminal domain, the N-terminal P/V region of V protein contributes to counteracting host cell antiviral

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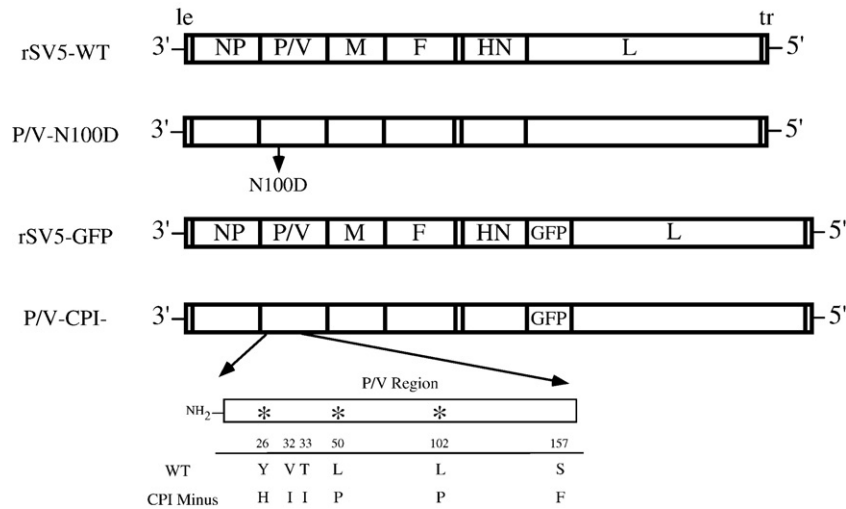


Fig. 1. Schematic diagram of SV5 viruses used in this study. The genome structures of rSV5-WT, P/V-N100D, WT rSV5-GFP and P/V-CPI- are shown schematically as negative sense RNA. Only rSV5-GFP and P/V-CPI- encode an additional gene (GFP) inserted between HN and L. The P/V-N100D and P/V-CPI- mutants encode a single N100D mutation or a set of six CPI- mutations in the shared P/V N-terminal region, respectively. The differences between the WT and CPI- P/V region is shown at the bottom with stars denoting the residues shown previously to be important for targeting STAT1 for degradation (Chatziandreou et al., 2002). le, leader; tr, trailer.

pathways (Chatziandreou et al., 2002; Wansley and Parks, 2002). This is evident from the naturally-occurring CPI- variant of SV5 which is defective in targeting STAT1 degradation and in blocking IFN signaling (Chatziandreou et al., 2002). Mutational analyses have identified amino acid differences in the P/V region between WT SV5 and CPI- that are responsible for defects in targeting STAT1 for degradation (Chatziandreou et al., 2002). We have previously engineered a recombinant rSV5 mutant (P/V-CPI-) to encode these same six CPI- P/V

substitutions in the background of the WT rSV5 genome (Wansley and Parks, 2002). Our analyses have shown that in human cells, these P/V gene substitutions converted WT rSV5 into a mutant that could not induce loss of STAT1 as expected, but the P/V mutant was also found to be a potent inducer of IFN and proinflammatory cytokines (Wansley and Parks, 2002; Young et al., 2006).

WT SV5 is unable to effectively block IFN signaling in mouse cells, and this is due at least in part to the V protein being altered in its

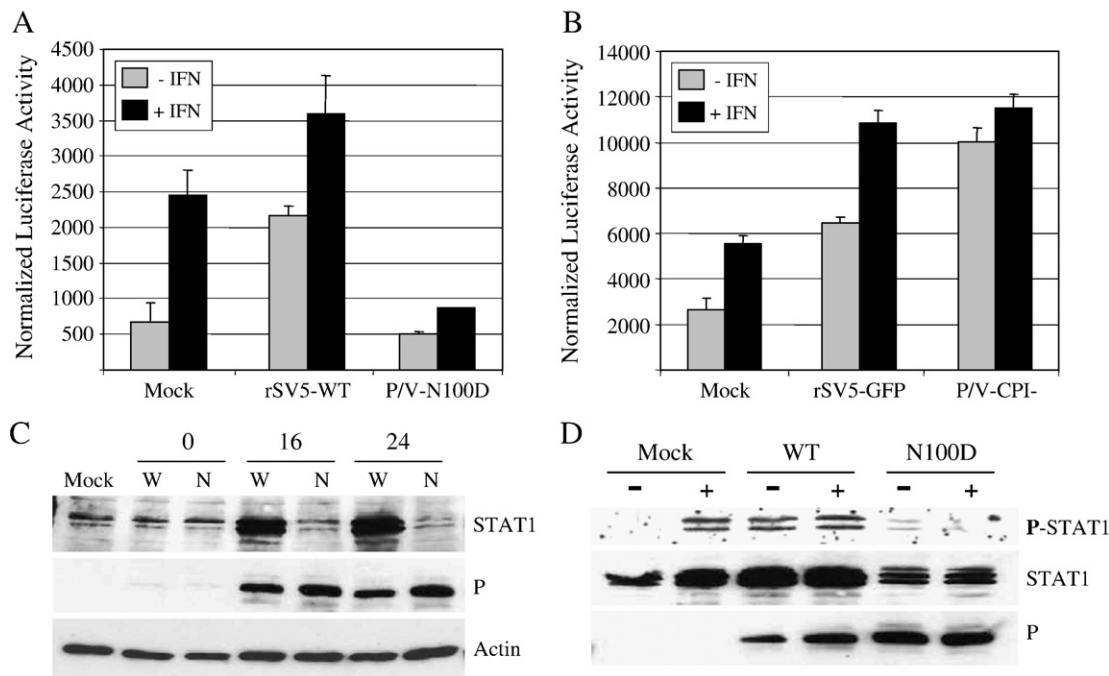


Fig. 2. WT and P/V mutant SV5 with differential ability to block IFN signaling in mouse cells. (A and B) Differential activation of ISRE-luciferase. Mouse CL7 cells were co-transfected with pSV-beta-gal and a plasmid containing the luciferase gene under control of an ISRE. Twenty-four hours post-transfection, cells were mock infected or infected at an moi of 20 with WT rSV5 or rSV5-P/V-N100D (panel A) or with rSV5-GFP or rSV5-P/V-CPI- (panel B). At 15 h pi, cells were incubated for 6 h with (black bars) or without (grey bars) 1000 U of mouse IFN-beta, after which lysates were prepared and luciferase and Beta-gal assays were performed. Normalized luciferase activities are representative of three independent experiments with bars representing standard deviation from the mean. C) STAT1 levels in P/V-N100D infected cells. CL7 cells were mock infected or infected at high moi with rSV5-WT (W lanes) or P/V-N100D (N lanes). Cell lysates were prepared at the indicated times pi and analyzed by Western blotting for levels of STAT1, viral P protein and cellular actin. D) STAT1 phosphorylation. CL7 cells were mock infected or infected at high moi with the indicated viruses. At 24 h pi, cells were treated with (+) or without (-) 1000 U of mouse IFN-beta for 6 h. Cell lysates were analyzed for STAT1, P protein and STAT1 phosphorylated on tyrosine 701 (P-STAT1).

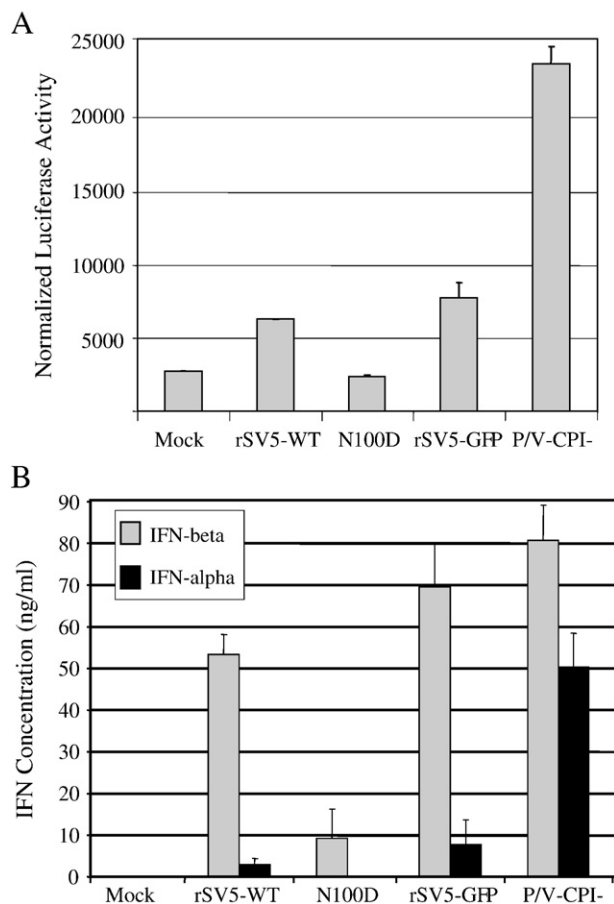


Fig. 3. Differential induction of IFN in mouse cells infected with WT rSV5 and P/V mutants. A) Induction of the IFN- β promoter. CL7 cells were co-transfected with pSV-beta-gal and a plasmid containing the luciferase gene under control of the IFN- β promoter. Twenty-four hours post-transfection, cells were mock infected or infected at high moi with the indicated viruses. Cells were lysed at 15 h pi, and normalized luciferase activity was calculated as in Fig. 2. Data are the average of three experiments with bars indicating standard deviation from the mean. B) IFN ELISA. CL7 cells were infected at high moi with the indicated viruses, and media collected at 24 h pi were analyzed by ELISA for levels of IFN- β (grey bars) or IFN- α (black bars). Results are the average of three experiments with bars indicating standard deviation from the mean.

interaction with mouse STAT2, a required component for STAT1 degradation (Horvath, 2004). During serial passage of SV5-infected mouse tissue culture cells, a viral isolate was identified (mci-2) which had gained the ability to block IFN signaling (Young et al., 2001). The genetic basis for this new phenotype was shown to be a single point mutation in the shared P/V N-terminal domain at position 100 that changes an asparagine residue to aspartic acid (N100D). While the mechanism by which the P/V-N100D mutant blocks mouse IFN signaling was not previously reported, this mutant virus grew to higher titers than the WT virus in mouse culture cells.

The striking differences in cell culture phenotypes of the P/V-CPI- and the P/V-N100D mutants compared to WT rSV5 raised the question of whether these viruses would elicit differences in adaptive immune responses in animals. Our panel of SV5 variants represents viruses that are poor inducers (WT rSV5 and P/V-N100D) and potent inducers (P/V-CPI-) of antiviral pathways. Given the role of IFN as an adjuvant (Bracci et al., 2006; Braun et al., 2002; Fink et al., 2006), we hypothesized that SV5 variants that induce higher levels of host cell responses would elicit higher adaptive immune responses, such as anti-SV5 antibodies. However, given the role of IFN as an inhibitor of virus replication (Didcock et al., 1999a; Goodbourn et al., 2000), an alternative hypothesis was that the variants which elicit higher levels of host cell responses would be restricted for growth, which could in turn lead to lower antibody titers (Atmar et al., 2007; Braciale et al., 1976; Mak et al., 1982).

Here we have utilized the mouse and ferret animal model systems to test these two hypotheses by determining the magnitude and kinetics of the antibody response to respiratory infection with WT rSV5 and P/V mutants.

Results

In vitro growth properties of WT and P/V mutant SV5 viruses in mouse cells

As diagramed in Fig. 1, we employed two matched pairs of WT SV5 and P/V mutant viruses which differ in their ability to limit host cell responses. One pair consisted of the P/V-N100D mutant virus, which can suppress IFN signaling in murine cells (Young et al., 2001), and WT rSV5, which cannot block IFN signaling in mouse cells as the proper control. The second pair consisted of the P/V-CPI- mutant, which is a potent inducer of antiviral responses, and a WT rSV5 encoding GFP as an additional transcription unit between HN and L, which was the proper control virus to account for the GFP gene encoded by P/V-CPI- (Fig. 1; Wansley and Parks, 2002). This additional gene has been shown previously to have no detectable effect on rSV5 growth properties (He et al., 1997).

To determine the relative ability of WT and the two P/V mutants to modulate IFN-I signaling, mouse CL7 fibroblast cells were transfected with a luciferase reporter gene plasmid under control of an ISRE and then infected at high moi with WT rSV5 or one of the P/V mutants. Fifteen hours pi, cells were challenged with 1,000 units of IFN- β , and luciferase activity was assayed at 6 h post-challenge. As shown in Fig. 2A, mock-infected cells responded to IFN challenge by increasing luciferase activity (black bar). Cells infected with rSV5-WT also responded to IFN challenge with slightly higher levels of luciferase activity, but cells infected with the P/V-N100D mutant were largely non-responsive to IFN

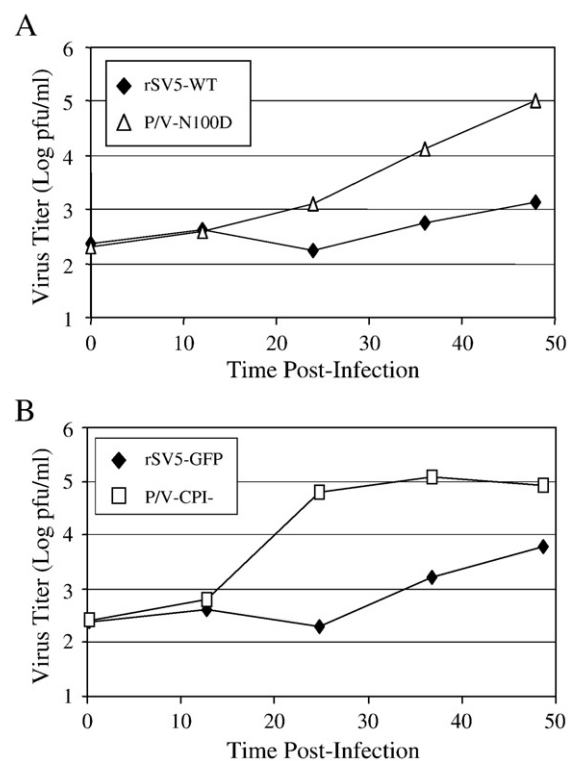


Fig. 4. Multicycle growth of WT and P/V mutant SV5 in mouse CL7 cells. Mouse CL7 cells were infected at an moi of 0.05 with the indicated WT rSV5 or P/V mutants. At the indicated times pi, media were collected and assayed for infectious virus by plaque assay. Data are the average of three independent experiments.

stimulation (Fig. 2A) as previously reported (Young et al., 2001). As shown in Fig. 2B, cells infected with the P/V-CPI- virus showed elevated IFN- β signaling with or without added IFN- β , similar to that reported previously for P/V-CPI- infection of human epithelial cells (Wansley and Parks, 2002).

The status of STAT1 in mouse cells infected with our WT rSV5 and P/V mutant viruses was analyzed. CL7 cells were infected at high moi with rSV5-WT or P/V-N100D, and cell lysates were analyzed at different times pi by Western blotting. As shown in Fig. 2C, cells infected with rSV5-WT had higher levels of STAT1 than mock-infected cells, consistent with IFN induction and signaling pathways being activated. By contrast, infection with the P/V-N100D mutant did not significantly change STAT1 levels compared to that seen for mock-infected cells. Immunofluorescence microscopy confirmed that all cells were infected by the P/V-N100D virus at the high moi used in these experiments (not shown).

Infection with the P/V-N100D virus blocked the ability of exogenously-added IFN- β to activate STAT1 phosphorylation. This is evident in Fig. 2D, where treatment of mock-infected or rSV5-WT-infected CL7 cells with IFN- β resulted in phosphorylation of STAT1

on tyrosine 701, a critical modification for STAT1 activation (Shuai et al., 1993). By contrast, treatment of P/V-N100D-infected mouse cells with IFN- β did not lead to increased STAT1 phosphorylation. Together, these data support previous reports (Young et al., 2001) that mouse cells infected with the P/V-N100D virus are defective in response to IFN, but extend those findings to show that the P/V-N100D mutant blocks STAT1 phosphorylation by a mechanism that does not include targeted degradation.

The data in Figs. 2A and B (grey bars) show that murine cells infected with rSV5-WT, rSV5-GFP or the P/V-CPI- mutant have activated IFN signaling pathways, even in the absence of exogenously-added IFN. To test the hypothesis that infection with these viruses induced IFN, CL7 cells were transfected with a luciferase reporter gene plasmid under control of the IFN- β promoter and infected at high moi with the viruses indicated in Fig. 3A. Levels of luciferase were determined at 15 h pi. Infection with the two viruses encoding a WT P/V gene (rSV5-WT and rSV5-GFP) resulted in a ~2–3 fold increase in IFN- β promoter activity, whereas cells infected with the P/V-CPI- virus showed nearly a 10 fold increase. By contrast, cells infected with the P/V-N100D mutant showed luciferase levels that

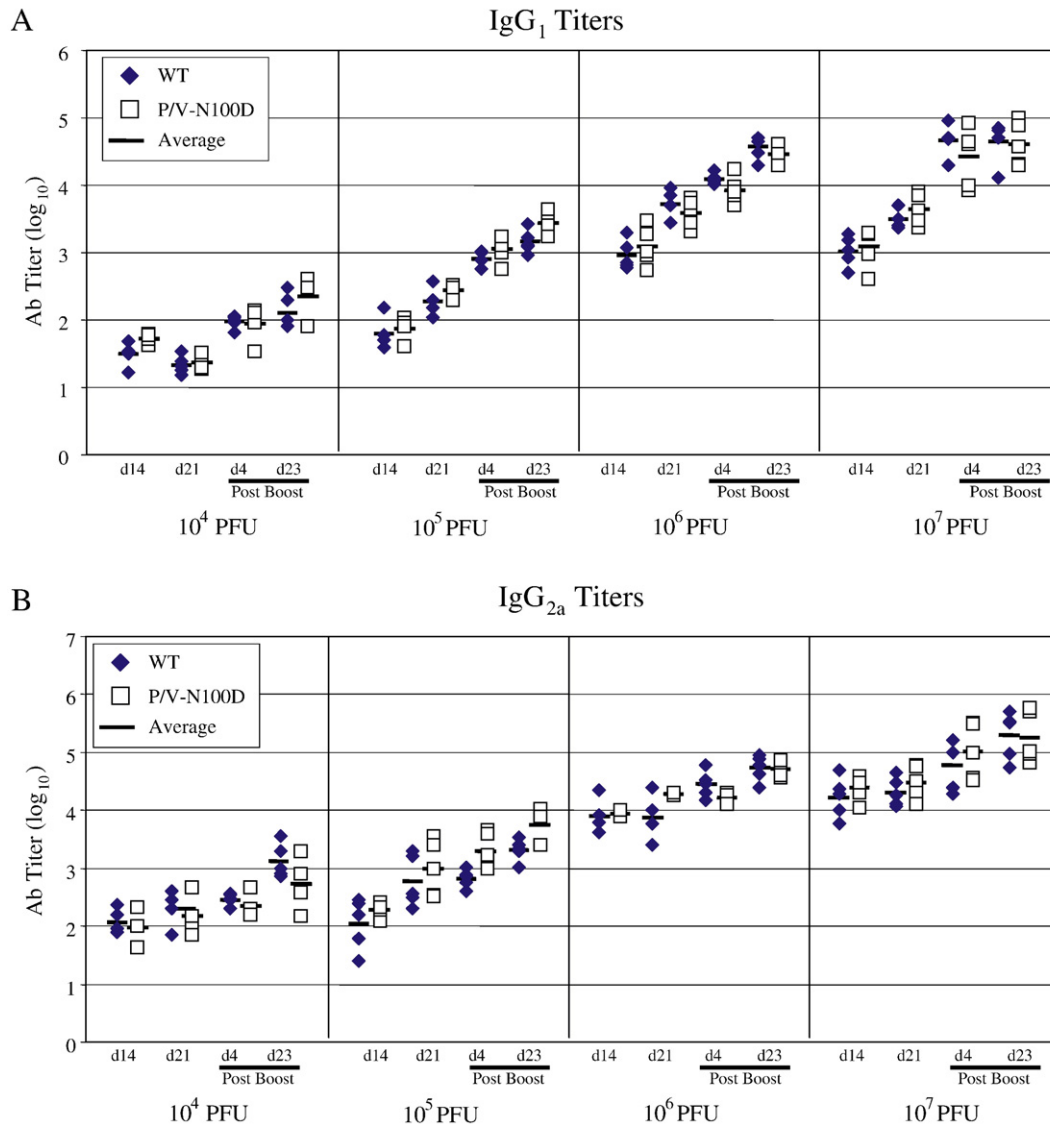


Fig. 5. Effect of virus dose on antibody responses in mice infected with WT SV5 and P/V-N100D. Groups of 5 BALB/c mice were infected i.n. with the indicated doses (in pfu) of WT rSV5 (closed diamonds) or P/V-N100D (open squares). At days 14 and 21 pi sera were collected and assayed by ELISA for anti-SV5 IgG₁ (panel A) and IgG_{2a} (panel B) responses. Mice were boosted with an equivalent amount of homologous virus, and levels of anti-SV5 antibodies were determined at day 4 and day 23 post boost. Each symbol represents data for an individual mouse with the horizontal bar indicating the mean value for the group.

were indistinguishable from those seen with mock-infected cells. These results were supported by ELISA data, in which levels of secreted IFN- β and IFN- α were determined at 24 h pi. As shown in Fig. 3B, the P/V-CPI- mutant induced slightly higher levels of IFN- β than either the rSV5-WT or rSV5-GFP viruses. However, the P/V-N100D mutant was a poor inducer of IFN- β . The P/V-CPI- mutant was the only virus that induced high levels of IFN- α .

Taken together, these results demonstrate that WT SV5 and the P/V mutants fall into two groups: 1) the P/V-N100D mutant virus, which is a poor inducer of IFN and limits IFN signaling, and 2) the P/V-CPI- mutant, which, like WT rSV5-GFP, is defective in blocking IFN signaling, but which additionally is a potent inducer of IFN, as it induces IFN to higher-than-WT levels. As predicted by these properties, the P/V-N100D virus grew in CL7 cells to titers that were approximately 2 logs higher in multi-step growth assays (Fig. 4A). Unexpectedly however, the P/V-CPI- virus grew to higher titers than rSV5-GFP (Fig. 4B), despite being a more potent activator of IFN responses. This enhanced growth may be a consequence of higher levels of viral gene expression and genome replication as described previously in human epithelial cell lines (Wansley and Parks, 2002).

Virus growth and antibody responses to i.n. infection of mice with WT or P/V mutant SV5

Based on our *in vitro* data above, we tested the hypothesis that intranasal (i.n.) infection of mice with these mutant viruses would elicit different antibody titers. Groups of five mice each were infected i.n. with increasing doses (10^4 , 10^5 , 10^6 or 10^7 pfu) of purified WT rSV5 or P/V mutant viruses and boosted with an equivalent amount of the homologous virus on day 21 pi. Infected animals showed no clinical signs of illness and pathological lesions in the nasal cavities were mild, consisting of infiltration by mixed type inflammatory cells, superficial epithelial injury and edema (data not shown). At the days pi shown in Fig. 5, sera were collected and used in an SV5-specific ELISA to determine the titers of IgG₁ and IgG_{2a}. These isotypes were chosen because they are indicative of the CD4⁺ T_H cell response to virus infection (Finkelman et al., 1990), and therefore provide an internal control to our experiment that would ensure we were not missing any portion of the antibody response because of a missing T_H1 or T_H2 population. There was a dose-dependent increase in the anti-SV5 IgG₁ (Fig. 5A) and IgG_{2a} (Fig. 5B) isotype response following i.n. infection of mice with doses between 10^4 and 10^6 pfu, and the highest dose of virus yielded final titers that were nearly 100-fold higher than the low dose of virus. A dose of 10^7 pfu did not significantly increase responses over that seen with 10^6 pfu. Most importantly however, there was no difference in antibody titers elicited with the WT rSV5 parental virus and P/V-N100D virus. Similarly, there was no difference in the antibody responses elicited by rSV5-GFP compared to the P/V-CPI- mutant (data not shown).

We tested the hypothesis that the similar antibody response in mice to WT and P/V mutant infection was due to similar *in vivo* growth. Groups of BALB/c mice were infected i.n. with 10^6 pfu of virus, and viral titers in tissues from the lungs were determined at various times pi. As shown in Fig. 6A, mice infected with rSV5-WT or the P/V-N100D mutant virus showed high titers of virus at early times pi, with a plateau between 3 and 7 days pi. A very similar profile of virus titers was seen in the case of mice infected with the rSV5-GFP and P/V-CPI- mutant viruses (Fig. 6B). WT rSV5 and P/V mutants grew to higher titers and virus was recovered at later times pi in nasal tissues compared to lung tissues; however, there were no differences in virus growth between animals infected with either virus (Fig. 6C). Together, these data indicate that infection of mice with rSV5 viruses that differ in their ability to induce IFN does not result in differences in levels of virus recovered from the respiratory tract, and there is no dose-dependent difference in anti-SV5 antibody responses elicited in mice by the WT and P/V mutants.

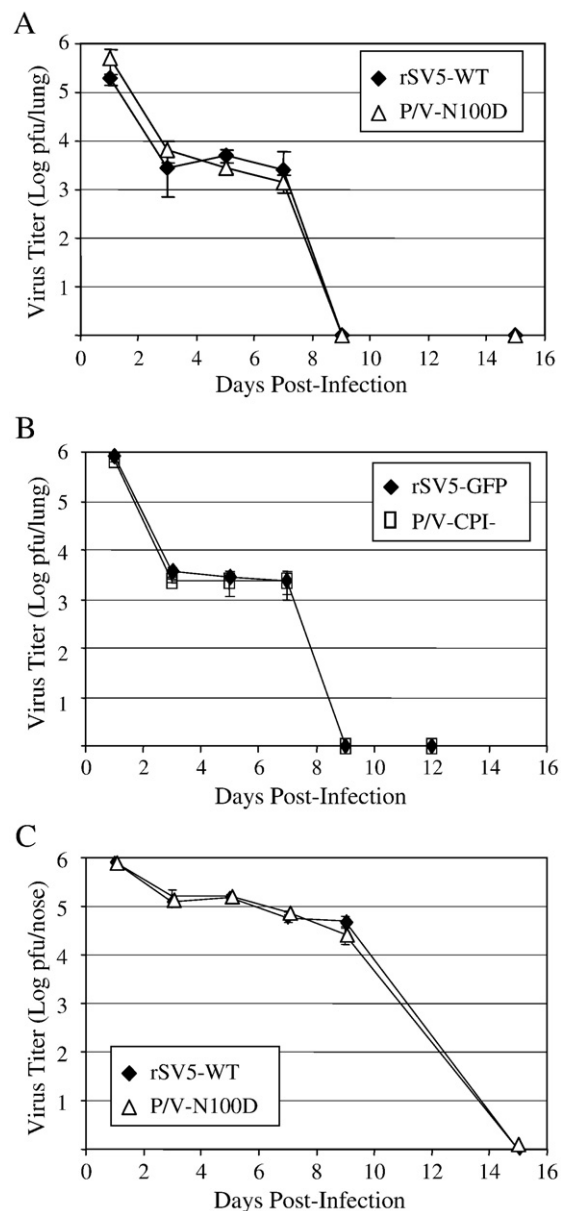


Fig. 6. Growth of WT and P/V mutant rSV5 in mice. Groups of BALB/c mice were infected i.n. with 10^6 pfu of rSV5-WT or P/V-N100D (panels A and C) or with rSV5-GFP or P/V-CPI- (panel B). At the indicated days pi, lungs (panels A and B) or nasal tissue (panel C) were harvested and assayed for infectious virus by plaque assay as described in Materials and methods. Data are the average of three mice per time point, with bars representing the standard deviation from the mean.

Growth properties of rSV5-GFP and P/V-CPI- viruses in primary cultures of ferret lung fibroblasts

It has been previously reported that SV5 is restricted for growth in mice (Parisien et al., 2002; Young et al., 2001). As an alternative animal model to test our hypotheses, we turned to the ferret system, which has been used as a model for studying a number of respiratory virus infections (Durchfeld et al., 1991; von Messling et al., 2003; Zitzow et al., 2002). In this model system, the P/V-N100D mutant virus becomes irrelevant, since this virus has phenotypes that are specific for the mouse system. Primary cultures of lung fibroblasts were established from naïve ferrets as described in Materials and methods. Staining with anti-cytokeratin antibodies showed these cells were >95% fibroblasts (data not shown). To determine the growth properties of WT SV5 and P/V-CPI- in ferret cells, cultures

were infected at high moi and examined by western blotting for NP and GFP expression. As shown in Fig. 7A, very low levels of NP and GFP were detected at 12 h pi, but were greatly enhanced by 24 h pi. In multi-step growth analyses, the P/V-CPI- mutant grew to a final titer that was ~1 log lower than rSV5-GFP (Fig. 7B), consistent with our previous results with human tissue culture cell lines (Wansley and Parks, 2002). It is noteworthy that titers of both WT and P/V mutant SV5 in the fibroblast cells shown in Fig. 7B were ~2 logs lower than that seen in human epithelial cells (Wansley and Parks, 2002). This is consistent with our findings that SV5 yields in many fibroblast cell lines are typically much lower than that seen in epithelial cells (data not shown).

To determine the effect of WT SV5 and P/V-CPI- infection on ferret STAT1, ferret cells were infected at high moi with rSV5-GFP or P/V-CPI-, and cell lysates were analyzed by Western blotting for levels of STAT1. Consistent with our previous results from human cells (Wansley and Parks, 2002), WT infection induced the rapid degradation of STAT1, and by 12 h pi there was little detectable STAT1 (Fig. 7C). By contrast, ferret cells infected with the P/V-CPI- mutant virus showed no significant loss of STAT1. STAT1 was activated following infection of ferret cells with P/V-CPI- but not WT SV5. This is evident in Fig. 7D where cell lysates were analyzed by Western blotting for phosphorylation of STAT1 on residue tyrosine 701 as an indication of IFN signaling. These data are consistent with in vitro results with human cell lines, and suggest that IFN is induced by infection with the P/V-CPI- mutant.

An RPA was carried out to determine if ferret IFN-beta was differentially induced by WT rSV5 and the P/V-CPI- mutant. A cDNA encoding ferret IFN-beta was generated by RT-PCR using RNA from

naïve ferret lung fibroblasts, as described in Materials and methods. RNA was harvested at 20 h pi with WT rSV5-GFP or the P/V-CPI- mutant and was analyzed using a negative sense ³²P-labeled ferret IFN-beta riboprobe. As shown in Fig. 7E, samples from P/V-CPI- infected cells yielded a protected band consistent with IFN-beta mRNA expression, while no signals were seen in the case of RNA from mock-infected or rSV5-GFP infected cells. These results are consistent with previous results obtained in human cells showing induction of IFN-beta expression by the P/V-CPI- mutant (Wansley and Parks, 2002).

To determine the CPE following infection of ferret fibroblasts, cells were infected at high moi with rSV5-GFP or P/V-CPI- and examined at 48 h pi by microscopy. As shown in Fig. 8A, ferret cells infected with the rSV5-GFP virus showed very little CPE similar to that seen with mock-infected cells. By contrast, ferret cells infected with the P/V mutant virus showed extensive CPE. Cell viability assays (Fig. 8B) showed that cell cultures infected with rSV5-GFP had very little loss of viability. P/V mutant-infected fibroblasts showed a time-dependent loss of viability starting at ~48 h pi, and by 72 h pi very few viable cells were detected. The loss of viability in P/V-CPI- infected ferret cells correlated with increased levels of annexin V staining. This is evident in Fig. 8C, where rSV5-GFP infection resulted in a very low percentage of cells that were positive for annexin V staining. By contrast, ~65% of ferret cells infected with the P/V-CPI- mutant were positive for annexin V by 48 h pi. Taken together, the above data indicate that infection of primary cultures of ferret lung fibroblasts with WT and P/V mutant SV5 reproduces a number of phenotypes of infected human cells, including multi-step growth kinetics and differential activation of IFN pathways (Fig. 7), and apoptosis (Fig. 8).

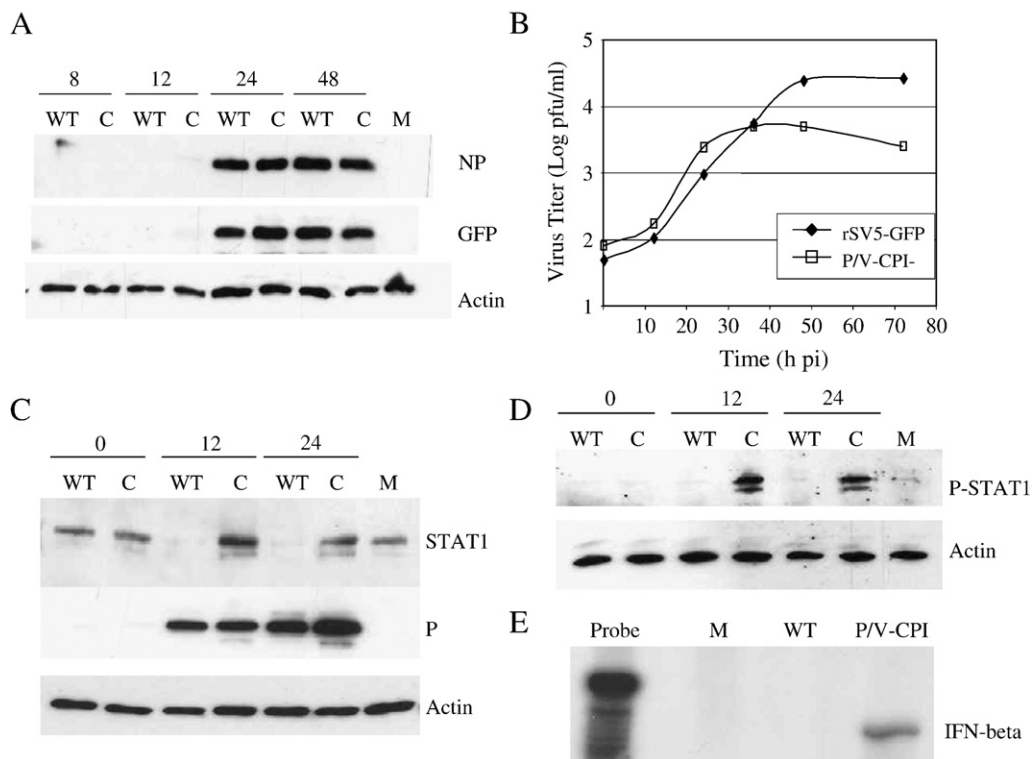


Fig. 7. In vitro growth properties of rSV5-GFP and P/V-CPI- mutant in ferret lung fibroblast cells. A) GFP expression. Primary cultures of ferret fibroblast cells were mock infected or infected at an moi of 10 with rSV5-GFP or P/V-CPI-. Cell lysates prepared at the indicated times pi were analyzed by western blotting for viral NP and GFP or for cellular actin. B) Low moi growth analysis. Ferret cells were infected with rSV5-GFP or P/V-CPI- at an moi of 0.05. Media were collected at the indicated times pi for analysis by plaque assay. Data are representative of four independent experiments. C and D) STAT1 status in ferret fibroblasts. Primary cultures of ferret fibroblasts were mock infected (M lane) or infected at an moi of 10 with WT rSV5-GFP (WT lanes) or the P/V-CPI- mutant (C lanes). At the indicated times pi, cell lysates were prepared for analysis by Western blotting for cellular STAT1, STAT1 phosphorylated on tyrosine 701 (P-STAT1), viral P protein or cellular actin as indicated. E) IFN- β gene expression. RNA was harvested at 20 h pi from ferret fibroblasts that were mock infected (M lane) or infected at an moi of 10 with rSV5-GFP (WT lanes) or P/V-CPI- and analyzed by RPA using a ³²P-radiolabeled anti-sense riboprobe (P lane) specific for ferret IFN-beta. Results are representative of three independent experiments.

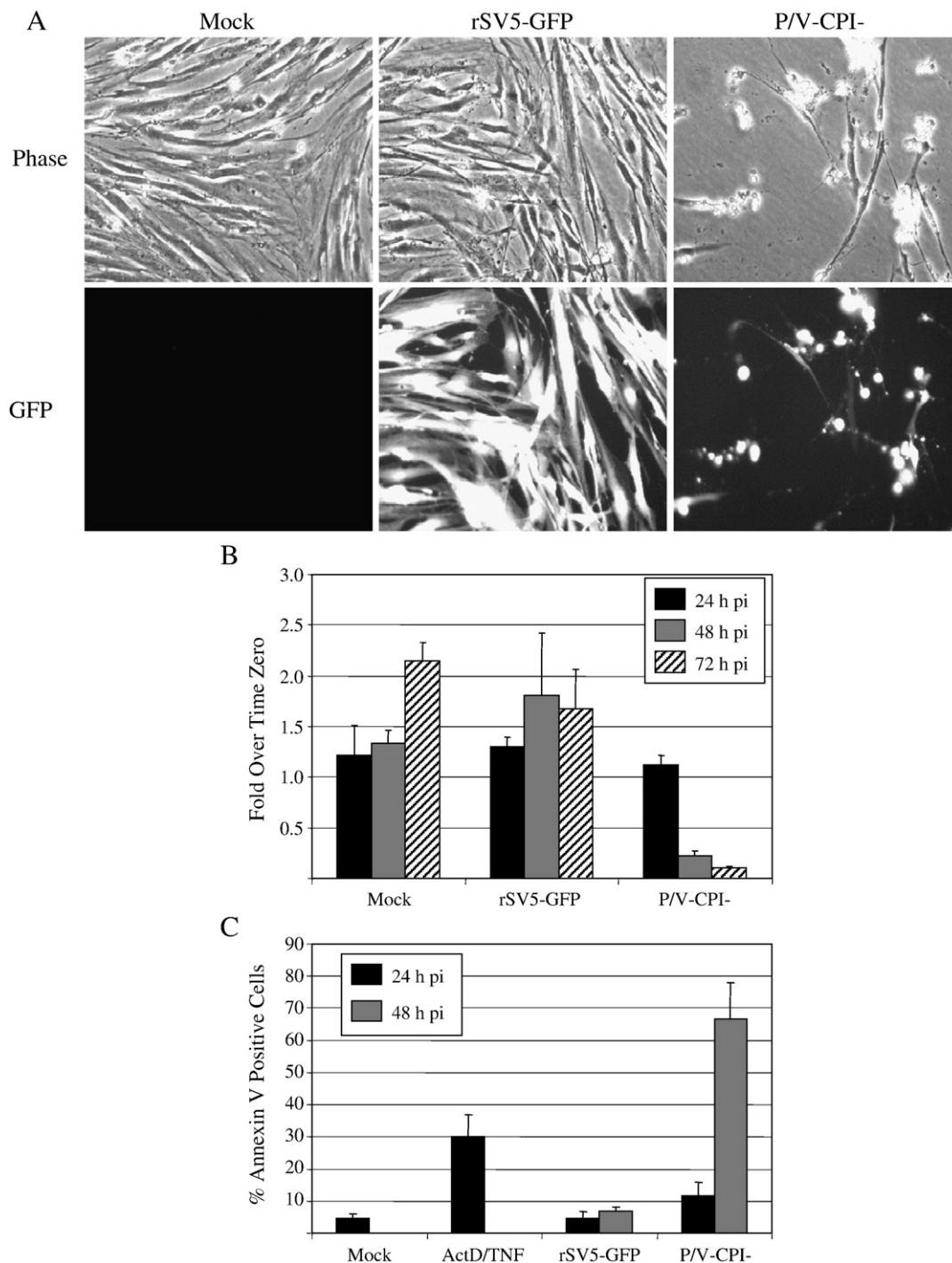


Fig. 8. Induction of CPE and apoptotic markers following infection of ferret fibroblasts with the P/V-CPI- mutant but not rSV5-GFP. A) Ferret fibroblasts were mock infected or infected at an moi of 10 with WT rSV5-GFP or the P/V-CPI- mutant. Cells were examined by microscopy at 48 hours pi for CPE and GFP expression. B) The number of viable cells at the indicated times pi was determined by trypan blue exclusion. Data are expressed as fold change in viable cell number compared to the starting number of cells and are the average of 3 samples per timepoint. Bars represent standard deviation from the mean. C) Cells were analyzed by flow cytometry at the indicated times pi for staining with PE-conjugated annexin V. Data are the average of 3 samples per timepoint, with bars representing the standard deviation from the mean.

Antibody responses to respiratory infection of ferrets with WT rSV5 and P/V mutant

Based on the above *in vitro* data in primary ferret fibroblasts, we tested the hypothesis that *i.n.* infection of ferrets with these viruses would elicit different antibody titers. Ferrets had no detectable preexisting antibodies that recognized SV5 in ELISAs (not shown). Groups of three ferrets each were infected *i.n.* with increasing doses

(10^4 , 10^6 or 10^8 pfu) of purified rSV5-GFP or P/V-CPI- virus and boosted with an equivalent amount of the homologous virus on day 28 pi. Infected animals showed no clinical signs of illness, and pathological lesions in the nasal cavities included minimal sub-mucosal edema and small amounts of luminal hemorrhage. Significant lesions were not found in the lung or brain (data not shown). Thus, infection of ferrets with SV5 doses as high as 10^8 pfu was well tolerated.

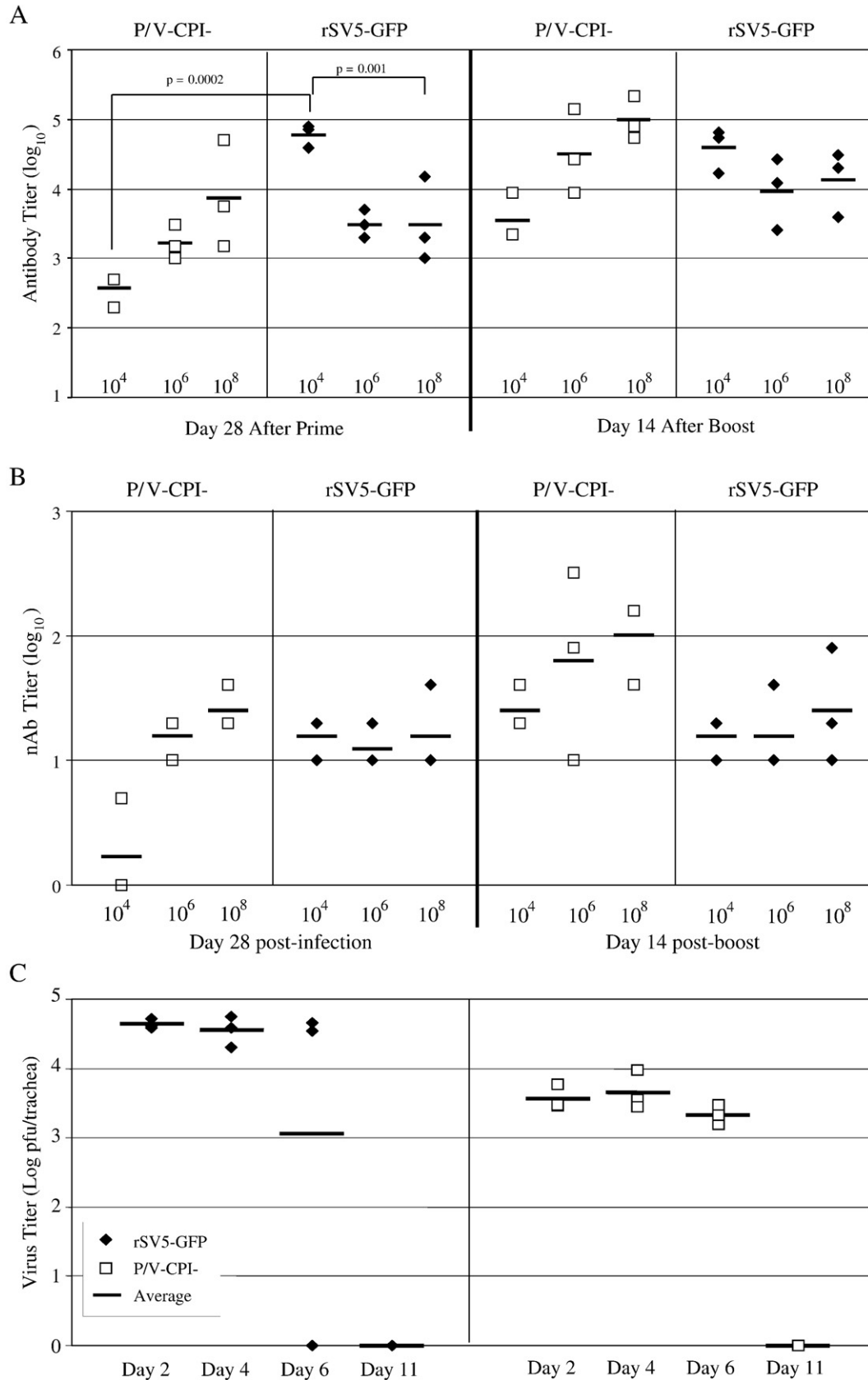


Fig. 9. Antibody response and viral titers elicited by intranasal infection of ferrets with rSV5-GFP and P/V-CPI-. A and B) ELISA and neutralizing antibody titers. Groups of 3 ferrets each were infected i.n. with 10^4 , 10^6 , or 10^8 pfu of rSV5-GFP or P/V-CPI-. At day 28 pi, serum was collected and ferrets were boosted with an equivalent amount of homologous virus. Levels of anti-SV5 antibodies in serum from day 28 and d14 post boost (panel A) and neutralizing titers in d28 and d14 post boost serum (panel B) were determined as described in Materials and methods. Each symbol represents data for an individual ferret with the horizontal bars indicating the mean value for the group. Results are representative of two independent experiments. C) Viral titers. Ferrets were infected i.n. with 10^4 pfu of rSV5-GFP or P/V-CPI-, and viral titers in the upper trachea of animals were determined at the indicated days pi. Each symbol represents the titer for one experimental animal, and the bar represents the mean value for the group.

Sera that were collected before boosting on day 28 pi and on day 14 post boost (pb) were assayed for levels of SV5-specific IgG by ELISA as described in the Materials and methods. As shown in Fig. 9A, ferrets that received the P/V-CPI- virus showed a dose-dependent increase in anti-SV5 antibody titers following the primary infection (left panel, open boxes), with mean titers reaching $\sim 10^4$ at the dose of 10^8 pfu. By contrast, ferrets that received rSV5-GFP (closed diamonds, Fig. 9A) showed the highest mean anti-SV5 titers with the lowest dose of virus in the primary infection (10^4). With increasing doses of rSV5-GFP there was a slight decrease in titer that was statistically significant ($p=0.001$). Most importantly, at the lowest dose of virus (10^4 pfu), rSV5-GFP elicited a mean antibody titer that was ~ 500 fold higher than that elicited with the P/V-CPI- virus, and this difference was statistically significant ($p=0.0002$). With increased doses of 10^6 and 10^8 pfu, the P/V-CPI- mutant elicited higher antibody titers, whereas rSV5-GFP elicited lower titers. Mean antibody titers to rSV5-GFP and the P/V mutant at 10^6 and 10^8 pfu were not statistically different. Thus, in the primary infection, a low dose of rSV5-GFP is more effective than the P/V-CPI- mutant at inducing anti-SV5 antibody responses. At higher doses, there is an increased response to P/V-CPI- and a slightly decreased response to rSV5-GFP such that the antibody responses are now equivalent.

Ferrets responded differently to boosting with WT rSV5-GFP and P/V-CPI- mutant. This is evident in Fig. 9A (right panels), where antibody responses to the P/V mutant increased significantly when animals were given a low or intermediate dose ($p<0.05$ for both doses). There was only a slight increase in antibody titers at the high dose, which was not statistically significant. By contrast, boosting with the WT rSV5-GFP virus did not significantly increase antibody titers. As shown in Fig. 9B, neutralizing Ab titers elicited by P/V-CPI- were slightly lower than WT rSV5-GFP; however, they did not differ significantly from each other at any dose tested.

To determine if the difference in ferret anti-SV5 responses at low dose correlated with differences in virus growth in vivo, groups of ferrets were infected i.n. with 10^4 pfu of rSV5-GFP or P/V-CPI-, and the trachea and lungs were assayed for virus levels by plaque assay at days 2, 4, 6, and 11 pi. No virus was detected in samples from the lower trachea or in ferret lungs (not shown). However, in samples from the upper trachea, viral titers from animals infected with rSV5-GFP were approximately one log higher than those seen for animals infected with the P/V-CPI- mutant (Fig. 9C). These data are consistent with the hypothesis that at the lowest dose, virus replication is higher for WT virus compared to the P/V mutant, which contributes to eliciting a higher anti-SV5 antibody titer.

Discussion

The goal of this study was to utilize the mouse and ferret animal model systems to test the hypothesis that SV5 P/V gene mutants which are more potent activators of cell responses in tissue culture will also be better at eliciting adaptive immune responses. This hypothesis was based on our previous work showing that: 1) the P/V-CPI- mutant was more effective than WT rSV5 at activating innate responses in cell culture (Wansley and Parks, 2002), and 2) human DC infected with the P/V-CPI- virus are more effective at activating T cell functions in vitro than DC infected with WT rSV5 (Arimilli et al., 2006). Likewise, it has been proposed that viruses encoding defective antagonists of antiviral responses would be more effective vectors for eliciting adaptive immunity (Talon et al., 2000; Valarcher et al., 2003). Here we show that primary cultures of ferret lung fibroblasts reproduce many of the in vitro human cell culture properties of WT SV5 and P/V-CPI- described previously, including differences in STAT1 degradation, induction of IFN synthesis and signaling, and CPE. Most importantly for our hypothesis, low dose intranasal infection of ferrets with WT and P/V mutant SV5 elicited different antibody responses, and different levels of virus were recovered from the ferret trachea. By contrast, in the mouse model system WT and P/V mutants showed differences in their

ability to counteract IFN responses in tissue culture cells, but intranasal infection of mice with these mutants did not result in differences in either virus growth or antibody responses at any dose tested. Together, these data support the proposal that ferrets represent an excellent animal model system for in vivo analysis of SV5 growth and immune responses.

SV5 infection efficiently targets STAT1 for degradation in human cell lines (Didcock et al., 1999b; Young et al., 2000), and in tissue culture cells from a number of other species including monkey, dog, and horse, but not rabbit cells (Precious et al., 2005). In our primary cultures of ferret cells, WT SV5 efficiently targeted ferret STAT1 for degradation, whereas the P/V-CPI- mutant which is defective for this property in human cells (Wansley and Parks, 2002) is also defective in STAT1 degradation in ferret cells. Furthermore, our results show that the P/V-CPI- mutant virus induced higher levels of IFN-beta mRNA than WT rSV5, consistent with this mutant virus being defective in limiting IFN induction in ferret cells. The inability to limit IFN induction and signaling pathways likely contributes to the P/V-CPI- mutant virus having attenuated low moi growth in ferret cells compared to WT SV5, a property also seen during infections of human tissue culture cells (Wansley et al., 2005). These findings raised the hypothesis that differences in the ability of WT SV5 and P/V-CPI- to limit ferret cell antiviral responses would be reflected in vivo by differences in adaptive responses to infections of the ferret respiratory tract.

The ferret is a widely-used animal model for studying viral infections (Rudd et al., 2006; Silin et al., 2007; Svitek and von Messling, 2007; von Messling et al., 2006; Yen et al., 2007). Ferrets are highly susceptible to a number of experimental viral respiratory infections, including influenza virus (Zitzow et al., 2002), canine distemper virus (CDV; Hoover et al., 1989; Svitek and von Messling, 2007), and SARS-associated corona virus (Martina et al., 2003). In the case of CDV, infected ferrets show massive dissemination of virus throughout the body, with excessive morbidity and mortality (von Messling et al., 2003). In our studies, infection of the ferret respiratory tract with WT SV5 or the P/V-CPI- mutant resulted in no overt indication of illness – animals remained playful with no changes in either body weight or temperature. Likewise, analyses of respiratory tract tissues did not provide any evidence of virus-induced pathology (data not shown). These results are consistent with previous work examining the pathology of the bone fide CPI virus in ferrets (Baumgartner et al., 1989, 1991), and with a mouse model system in which SV5 was only pathogenic under conditions in which immune responses were severely compromised, such as in STAT1-deficient mice (He et al., 2002; Randall and Young, 1991).

A low dose of only 10^4 pfu of WT rSV5 was sufficient to elicit a strong anti-SV5 antibody response, and virus was recovered from the trachea of infected animals up to 6 days after infection. For both WT and P/V mutant SV5, virus was detected in tissue from the ferret upper trachea, but there was no detectable virus in tissues from the lung or lower trachea as described previously for CPI infections of ferrets (Durchfeld et al., 1991). Importantly, at a low dose of 10^4 pfu the P/V-CPI- mutant elicited ~ 500 fold lower anti-SV5 antibody response than that seen with the same dose of WT virus. In addition, the antibody response to P/V-CPI- infection increased as the dose was increased between 10^6 and 10^8 pfu, whereas this was not seen with WT rSV5. Finally, with P/V-CPI-, the anti-SV5 antibody titer was increased by ~ 1 log following a secondary infection, whereas anti-SV5 responses were not altered following a booster infection with WT rSV5. Future work will focus on whether there is also a dose-dependent difference in cellular immune response to these two viruses, but this will require the development of appropriate ferret-specific reagents for cellular immunology.

Why is the antibody response to low dose infection with the P/V-CPI- mutant so much lower than that elicited by the WT SV5 virus? One of our original hypotheses was that the P/V-CPI- virus, which induces DC maturation and is defective in limiting cytokine induction

and counteracting IFN, would elicit higher-than-WT levels of antibodies, possibly due to the positive effects of innate cytokines on adaptive immune responses (Bracci et al., 2006; Braun et al., 2002; Fink et al. 2006). As an alternative hypothesis, the induction of innate responses to P/V-CPI- could limit virus replication, which in turn could limit the antibody responses. Our results are consistent with the second hypothesis, since i.n. infection with the P/V-CPI- mutant elicited lower antibody titers than that seen with WT rSV5 and lower levels of virus were recovered from the ferret respiratory tract. At higher doses of both viruses, we hypothesize that there is an increase in either the number or type of infected cells. In the case of the P/V mutant, this could overcome limitations in viral replication seen at the lower doses, resulting in higher antibody titers. With WT rSV5, we have shown that higher doses elicited slightly lower anti-SV5 antibodies, possibly due to different cell types being infected. Future work will focus on directly determining the levels of viral antigen and replication following infection with different doses of WT and P/V mutant SV5.

It is possible that ferret dendritic cells (DC) play a role in the antibody response to SV5 infection, since DC play a critical role in linking the innate and adaptive arms of the immune response to pathogens (Palucka and Banchereau, 1999). Our previous cell culture studies have shown that apoptosis is induced in human immature dendritic cells infected with WT rSV5, but not with the P/V-CPI- mutant (Arimilli et al., 2006, 2007). Likewise, DC infected with the P/V-CPI- mutant are more effective activators of T cell functions than DC infected with WT rSV5 (Arimilli et al., 2006). The role that DC plays in different ferret antibody profiles elicited by WT SV5 and the P/V mutant has not yet been evaluated. Anti-SV5 responses could be the result of DC acquiring antigen by direct infection or by cross-presentation. In the former case, differences in dose-dependent antibody responses to WT and P/V mutant SV5 could be explained by ferret DC being more readily infected *in vivo* at high rather than at low doses of virus. In the case of the P/V mutant, higher doses could lead to more infected DC and to a higher anti-SV5 response. In the case of WT SV5, higher doses could lead to virus-induced apoptosis of infected DC, and this could contribute to the slight decrease in antibody responses compared to the P/V-CPI- virus. Further experimentation is required to test this proposed mechanism involving ferret DC.

Interestingly, ferrets given a low dose of 10^4 pfu of P/V-CPI- showed lower total antibody responses compared to rSV5-GFP when assayed by ELISA, but the neutralizing titers elicited by the two viruses were not statistically different. This result may indicate that total antibody responses to SV5 proteins are higher in the case of WT rSV5-GFP consistent with higher replication, but antibody responses to surface HN and F are similar. Alternatively, levels of anti-HN or -F antibodies elicited by the P/V mutant may be low, but could be superior in their capacity for neutralization compared to higher levels elicited by rSV5-GFP, perhaps due to the adjuvant effect of cytokines elicited by the P/V mutant. Work is in progress to determine the anti-HN and anti-F responses elicited by WT and P/V mutant viruses, and the relative avidity of these antibodies.

In mouse cells, WT SV5 cannot target STAT1 for degradation (Young et al., 2001) and this is thought to be due to an inability of V protein to properly interact with murine STAT2 (Parisien et al., 2002; Precious et al., 2005). Most importantly for our hypotheses on innate and adaptive immunity, we have analyzed a previously identified SV5 P/V mutant (N100D) with a gain-of-function ability to block IFN signaling in mouse tissue culture cells (Young et al., 2001). We have extended this finding to show that the P/V-N100D virus prevents phosphorylation of STAT1 in response to exogenously-added IFN by an undetermined mechanism, but STAT1 levels do not appear to differ from that of mock-infected cells.

Here we show that the P/V-CPI- mutant is a very potent inducer of both IFN-beta and IFN-alpha in mouse cells, compared to the moderate induction of IFN-beta following WT rSV5 infection. Unexpectedly how-

ever, the P/V-N100D virus was found to be a poor inducer of IFN synthesis. Thus, the change of asparagine residue 100 to aspartic acid in the P and V proteins confers two new phenotypes on WT rSV5: a block in IFN signaling as shown by Young et al. (2001) and lower induction of IFN secretion. More detailed work is needed to uncover the mechanism by which the N100D mutation alters the ability of SV5 to limit IFN production. For the purposes of this study, our cell culture work identified a panel of WT and P/V mutants with decreasing ability to limit murine IFN induction and signaling pathways: N100D>WT rSV5>P/V-CPI-.

Our results with antibody responses to WT and P/V mutant SV5 in the mouse system differ significantly from that of the ferret system. Following i.n. infection of mice, anti-SV5 antibody responses were similar between WT SV5 and each of our P/V mutants at all doses tested. Likewise, there was no detectable difference in levels of virus recovered from mice infected with any of the SV5 viruses. These data indicate that there are restrictions on virus growth in the murine respiratory tract that are not evident in our cell culture system, and that they are independent of any differences in P/V mutations. It is unlikely that these restrictions are due exclusively to IFN, since the growth of WT SV5 and P/V-CPI- in mice deficient in the IFN receptor (IFNAR-/- mice) was very similar and was not significantly enhanced over normal mice (data not shown). In this regard, there may be species specific restrictions in SV5 replication in murine cells, since the yield of SV5 progeny from CL7 mouse tissue culture cells is ~100–1000 fold lower than that typically seen with human cell lines *in vitro*.

Kraus et al. (2008) have recently reported a transgenic mouse system in which WT rSV5 can induce STAT1 degradation and block IFN signaling. In these human STAT2 transgenic (hSTAT2 Tg) mice, WT rSV5 grows to ~100 fold higher titer in the respiratory tract compared to normal control mice (Kraus et al., 2008). Why is virus replication enhanced in the case of WT SV5 infection of hSTAT2 Tg mice compared to normal mice, but the N100D mutant, which is a poor inducer of IFN and blocks IFN signaling, grows to titers that are similar to WT rSV5? One possibility is that there may be added benefit to virus replication by degrading STAT1 in the case of WT rSV5-infected hSTAT2 Tg mice versus blocking STAT1 phosphorylation as our data indicate for the P/V-N100D mutant. In this regard, there is evidence that STAT1 may serve as a constitutive transcription factor for basal expression of some cellular genes (reviewed in Horvath, 2000), and virus replication *in vivo* may be enhanced when this function is eliminated due to STAT1 degradation. A second possibility is that lymphocytes recruited to the site of infection in hSTAT2 Tg mice may differ in their capacity to mount antiviral responses compared to control normal mice. An analysis of the effector functions of lymphocytes in the hSTAT2 Tg mouse and P/V-N100D systems may shed light on this proposal.

In summary, at low doses an SV5 P/V gene mutant that is a potent inducer of IFN in tissue culture cells grows to lower titers and is less effective than WT SV5 in eliciting antiviral antibody responses in the ferret model system. This contrasts with results from the mouse model system where P/V mutants and WT SV5 show no differences in growth in the respiratory tract or in anti-SV5 antibody responses, despite their abilities to induce IFN. However, in both systems, the level of *in vivo* growth correlated with the level of antibody produced.

Materials and methods

Cells, viruses, growth analysis and plaque assays

Mouse CL-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Primary lung cell cultures were made from naïve sable ferrets. The lungs were excised and washed three times in sterile phosphate-buffered saline (PBS). Lungs were minced into small pieces (~1 mm), and FBS was added drop-wise to each piece. Minced lung pieces were mashed and cultured for 4 h at 37 °C, followed by the addition of DMEM containing 20% FBS, and further culturing until growth to confluence. To determine the percent of

fibroblasts in these primary cell cultures, cells were stained with an anti-vimentin antibody (Santa Cruz Biotechnology) followed by staining with an anti-goat IgG antibody conjugated to AlexaFluor 468 and visualized by fluorescent microscopy.

A WT rSV5 and rSV5 expressing GFP (He et al., 1997) were recovered as described previously (Parks et al., 2002) from cDNA plasmids kindly provided by Robert Lamb (Northwestern University) and Biao He (Penn State University). The P/V-N100D mutant virus (Young et al., 2001) was provided by Biao He and was grown in MDBK cells. The P/V-CPI- mutant was recovered from cDNA and grown in Vero cells as described previously (Wansley and Parks, 2002). For low moi growth kinetics analyses, SV5 titers were determined by plaque assays on CV-1 cell monolayers as described previously (Wansley and Parks, 2002). For in vivo experiments, viruses were concentrated by centrifugation through a glycerol cushion (5 h; 25,000 RPM; SW28 rotor), and virus pellets were resuspended in a small volume of DMEM containing 0.75% BSA.

Transfection of reporter plasmids, IFN-I assays

Induction of the IFN-beta promoter in CL-7 cells was assayed using a luciferase reporter plasmid (pBlux) as described previously (Wansley and Parks, 2002). A pISRE-luc plasmid containing five copies of the ISG54 ISRE element upstream of a TATA box and luciferase reporter gene was used to measure induction of ISRE transcription. Six-well dishes of CL-7 cells were transfected as described above using 1 mg each of pISRE-luc and a β -galactosidase reporter plasmid. Twenty-four hours post-transfection, cells were mock-infected or infected at an moi of 20 with rSV5-WT or P/V mutants. Fifteen hours post infection (pi), culture media was supplemented for 6 h with or without 1000 U of mouse IFN- β (PBL Biomedical Laboratories). Cells were harvested in reporter lysis buffer, and normalized luciferase activity was calculated.

To directly assess the induction of mouse IFN, triplicate six-well dishes of CL-7 cells were mock infected or infected at an moi of 20 with rSV5 viruses. At 24 h pi, supernatants were harvested from infected cells, and IFN-alpha and IFN-beta levels in media were analyzed by ELISA as described by the manufacturer (PBL Biomedical Laboratories).

RNAse Protection Assay (RPA) for ferret IFN-beta gene expression

A ferret IFN-beta cDNA was generated from ferret fibroblast RNA using reverse transcription PCR with primers described previously (Svitek and von Messling, 2007). PCR products were cloned into a pGem7 plasmid and used to generate 32 P-labeled riboprobes for an RNAse protection assay (RPA) as described previously (Dillon and Parks, 2007). Briefly, six-well dishes of ferret lung fibroblasts were mock-infected or infected with rSV5-GFP or rSV5-P/V-CPI- at an moi of 10 in triplicate. At 20 h pi, total RNA was isolated in Trizol reagent. Following hybridization and RNAse digestion, samples were electrophoresed through a 6% polyacrylamide gel containing 9 M urea and analyzed by autoradiography.

SDS-PAGE, Western blotting, cell viability assays, apoptosis, and microscopy

For Western blotting, equivalent amounts of protein were loaded onto 10% gels, transferred to nitrocellulose membranes and analyzed with rabbit antisera to the SV5 P protein (Parks et al., 2002), cellular STAT1 protein (clone 554, Santa Cruz Biotechnology), STAT1 phosphorylated on tyrosine residue 701 (pSTAT, BD Biosciences), or actin, followed by HRP-conjugated secondary antibodies and visualization by enhanced chemiluminescence and exposure to film.

To quantitate SV5-induced cytopathic effect (CPE), triplicate six-well dishes of ferret lung fibroblasts were mock infected or infected with rSV5-GFP or P/V-CPI- at an moi of 10. At the indicated times pi, cells were trypsinized, and cell viability was determined by counting trypan blue-negative cells. To quantitate the levels of annexin V staining, cells were

mock infected or infected with rSV5-GFP or P/V-CPI- at an moi of 10. At the indicated times pi, cells were harvested with trypsin, stained with Annexin V conjugated to phycoerythrin (BD Biosciences), and analyzed using a FACScalibur flow cytometer as described previously (Arimilli et al., 2006). Microscopy experiments were performed on a Nikon Eclipse fluorescence microscope using a 20 \times lens. Images were captured using a QImaging digital camera and processed using QCapture software. Exposure times were manually set to be constant between samples.

Sections of lung and the entire heads of the ferrets were preserved in 10% neutral buffered formalin for at least 24 h. Following fixation, heads were decalcified in Decalcifier-2 solution overnight and coronal sections through the nasal cavity and calvarium, along with formalin fixed lung were embedded in paraffin, routinely processed for histology, cut at 4–6 μ m, stained with hematoxylin and eosin, and examined by light microscopy. The heads of the mice were treated similarly.

In vivo growth analysis and antibody responses

All animals were cared for in accordance with the policies of the Animal Care and Use Committee of Wake Forest University School of Medicine. Six to eight week old female BALB/c mice were purchased from Fredrick Cancer Research and Development Center (Fredrick, MD). For growth analyses, mice were infected intranasally (i.n.) with 10^6 pfu of purified rSV5-WT or P/V mutants in a volume of 10 μ l as described previously (Gray et al., 2003). At the indicated days pi, mice were sacrificed, and the lungs were harvested by dissection, snap-frozen and stored at –80 °C. Tissues were homogenized using a PowerGen 700 tissue homogenizer (Fisher Scientific) followed by clarification of tissue debris at 450 g for 10 min, and used in plaque assays as described above.

For antibody responses, mice were infected i.n. with the indicated doses of concentrated rSV5-WT or P/V mutants. Booster inoculations were carried out with an equivalent amount of homologous virus. At the indicated days pi, mice were bled from the tail vein, and the blood was allowed to clot overnight at 4 °C before clarification by centrifugation. For the SV5-specific ELISA, virus was purified by sucrose-gradient centrifugation as described previously (Paterson et al., 1995), and protein levels were determined by BCA. Plates were coated overnight at 4 °C with 12.5 μ g/ml of purified virus, and incubated with serial dilutions of mouse sera at room temperature for 2 h. After washing with PBS/Tween (0.02%), samples were incubated with HRP-conjugated anti-mouse IgG₁ or IgG_{2a} secondary antibodies for 1 h, followed by a final wash with PBS/Tween (0.02%) and incubation in the dark with TMB substrate solution (Sigma) before determining absorbance values at 450 nm. Final titers were expressed as the dilution of serum that yielded an absorbance of one-half the maximal absorbance obtained using a monoclonal antibody (4b) to SV5 HN protein run in parallel.

Four month old male ferrets were obtained from Marshall Farms (North Rose, NY) and infected i.n. with the indicated doses of purified rSV5-GFP or P/V-CPI- in a volume of 50 μ l. In vivo growth analyses were performed by harvesting the indicated regions of the respiratory tract and using the homogenized tissues in a plaque assay as described above. Ferret antibody responses were analyzed by withdrawing 1–3 ml of blood from the jugular vein on day 28 pi. Animals were boosted with an equivalent amount of homologous virus, and blood was collected by cardiac puncture at the termination of the experiment (day 14 post boost). ELISAs were performed using clarified sera as described above except the secondary antibody was an anti-ferret IgG (Bethyl Laboratories). Final antibody titers for ferret experiments were defined as the dilution of serum that yielded an absorbance of two times the absorbance obtained using serum from a naïve animal run in parallel.

To determine the levels of neutralizing antibody, clarified sera were serially diluted and incubated at room temperature for 30 min with 100 pfu of an rSV5 virus that expresses enhanced green fluorescence protein (rSV5-EGFP). This mixture was then added to MDBK cells and incubated at 37 °C for 1 h. The inoculum was replaced with fresh media,

and cells were incubated for 24 h, followed by analysis of EGFP expression by fluorescence microscopy. The neutralizing antibody (nAb) titer is defined as the lowest dilution of serum that yielded a 50% reduction in EGFP expression as compared to cells infected with 100 pfu of rSV5-EGFP in the absence of serum.

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